Identification and quantification of carotenoids from sarsaparilla (*Smilax aspera* L.) berries.

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Introduction

The sarsaparilla (*Smilax aspera* L.) is an evergreen perennial climbing plant of the *Liliaceae* family and typical of the Mediterranean basin. The plant grows and climbs from a rhizome and forms many extended branches - up to 15m in length - and numerous leaves around shrubs and trees. Stems are semi-woody and possess several prickles. The shiny leaves are generally heart-shaped with a few, tiny, translucent prickles along the margin. Flowers are associated as branched clusters. The fruits are juicy berries, which are initially green, turning red through ripening, sometimes becoming black. The berries are rather soft, having a spherical shape, 7-9mm across, and each holding a maximum of 3 seeds. The popularity of this plant is due to the ancient medicinal uses of the rhizomes, having depurative, diaphoretic, diuretic, stimulant and tonic properties (1, 2), so that they have been used as an ingredient in soft drinks. These therapeutic actions are mainly attributed to the high content in steroidal saponins (3).

Most of the attention has been given to the rhizomes of the *Smilax* genus, and no extensive works have been carried out on the chemical characterisation of the fruits. Recently the anthocyanin composition of *S. aspera* berries has been described as responsible of the fruit colour (4). However some preliminary studies in our laboratory have demonstrated that carotenoid pigments are also involved in the red coloration of these berries, and to our knowledge, the carotenoid composition of *S. aspera* berries has never been study and described. Therefore, the present work was aimed to isolate, identify and quantify the carotenoid pigments occurring in the *S. aspera* berries (5).

Figure 1. Fruits (A), flowers (B) and european geographical distribution (C) of sarsaparilla (*Smilax aspera* L.).

Materials and Methods

Raw material
Smilax aspera berries were collected during the autumn (October 2009) in a typical Mediterranean forest located at the Sierra Norte de Sevilla Natural Park (Sevilla, Spain; 37º37'44.3424'': -6º24'22.4316''). Samples were devoid of seeds and stored at -30ºC until analysis.

Carotenoid extraction
One gram of berries was extracted with acetone, until complete exhaustion of colour, according to the procedure of Mínguez-Mosquera and Hornero-Méndez (6). All extracts were pooled and transferred to diethyl ether. The ether phase, containing the carotenes and xanthophyll (free and esterified), was taken to dryness and the residue dissolved in 1 mL of acetone. When needed, a saponified extract was prepared by treating the direct extract with 10% KOH-MeOH. Extracts were kept at -30ºC until HPLC analysis.

HPLC analysis
Carotenoids were separated by using the HPLC protocol described by Mínguez-Mosquera and Hornero-Méndez (6) with slight modifications. Detection was carried out at 450 nm, and pigment were quantified in the direct extract by using calibration curves prepared with standard stock solutions in the concentration range 5-100 µg/mL.

Identification
Routine procedures for the identification of carotenoids have been used, which consisted of separation of pigments by TLC and co-chromatography with purified pigments, analysis of the UV-visible and mass spectra, and chemical test for 5,6-epoxide groups. Authentic pigment samples of β-carotene, lycopene, antheraxanthin, β-cryptoxanthin, zeaxanthin, lutein and rubixanthin were isolated and purified from natural sources (*Capsicum annuum*, *Rosa rubiginosa*, *Lycopersicum sculentum* and *Menta arvensis*). HPLC-MS(APcI) was used for determining the nature of the esterification moiety of xanthophyll esters by using the conditions Breithaupt and Schwack (7) with some modifications.

Fatty acids analysis
Fatty acids were extracted and analyzed by GC according to the method of Garcés and Mancha (8).

Results and Discussion

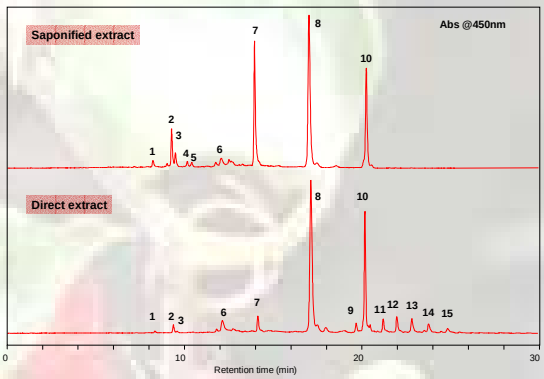

Figure 2. HPLC chromatograms corresponding to a direct and a saponified carotenoid extracts obtained from sarsaparilla (*S. aspera* L.) berries. (See **Table 1** for peak identities)

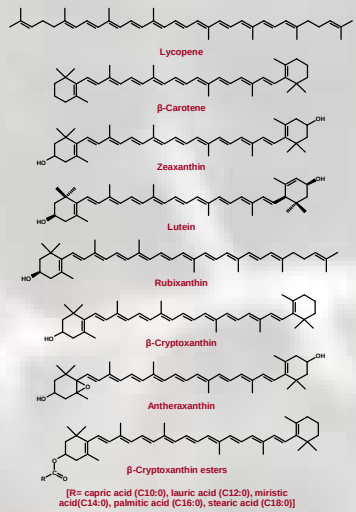

Figure 3. Chemical structures of the carotenoids identified in sarsaparilla (*S. aspera* L.) berries.

Table 1. Chromatographic, UV-visible and mass spectroscopy characteristics of carotenoids from sarsaparilla (*S. aspera* L.) berries. Quantitative analysis.

Peak	Carotenoid	Rt (min)	λ_{max} (nm)	λ_{max} (nm) according to bibliography in acetone	%III/II	Epoxide test	m/z [M+H]$^+$	m/z [M+H-FA]$^{+c}$	Concentration (µg/g fw)^d
1	*all-trans*-Antheraxanthin	8.33	427, 450, 479	425, 449, 478	57	+	585	-	0.58 ± 0.21
2	*all-trans*-Zeaxanthin	9.35	428, 455, 481	430, 452, 479	18	-	569	-	4.35 ± 0.78
3	*all-trans*-Lutein	9.56	423, 450, 476	424, 445, 474	62	-	569	-	0.68 ± 0.04
4	*9-cis*-Zeaxanthin	10.21	328, 426, 448, 478	330, 430, 452, 479	8	-	569	-	0.23 ± 0.06
5	*13-cis*-Zeaxanthin	10.44	332, 426, 448, 478	332, 430, 452, 479	14	-	569	-	0.26 ± 0.09
6	*all-trans*-Rubixanthin	12.01	440, 464, 494	440, 464, 494	51	-	553	-	42.00 ± 7.19
7	*all-trans*-β-Cryptoxanthin	14.17	430, 454, 480	428, 454, 480	18	-	553	-	7.42 ± 0.44
8	*all-trans*-Lycopene	17.90	448, 474, 505	448, 474, 505	66	-	537	-	242.44 ± 31.69
9	Zeaxanthin monoester[a]	18.74	430, 454, 481	430, 452, 479	38	-	n.d.[b]	n.d.	3.98 ± 0.37
10	*all-trans*-β-Carotene	20.68	427, 454, 479	429, 452, 478	18	-	537	-	65.76 ± 2.57
11	β-Cryptoxanthin caprate	21.65	428, 454, 478	428, 454, 480	19	-	707	535	7.34 ± 0.67
12	β-Cryptoxanthin laurate	22.42	428, 454, 479	428, 454, 480	24	-	736	535	8.88 ± 0.74
13	β-Cryptoxanthin myristate	23.24	428, 454, 479	428, 454, 480	23	-	763	535	8.86 ± 0.83
14	β-Cryptoxanthin palmitate	24.17	428, 452, 479	428, 454, 480	25	-	791	535	5.26 ± 0.11
15	β-Cryptoxanthin stearate	25.21	428, 452, 480	428, 454, 480	21	-	819	535	3.41 ± 0.32

a. Tentative identification; b. not detected; c. [M+H-FA]$^+$: Molecular mass of the ion after the neutral loss of the fatty acid moiety; d. mean ± standard deviation of a triplicate analysis

Figure 2 shows the HPLC chromatogram corresponding to the direct carotenoid extract obtained from fully ripe sarsaparilla (*S. aspera*) berries. When the extract was submitted to saponification with 10% KOH-MeOH the resulting chromatogram revealed the presence of xanthophyll acyl esters in the direct extract. With the aim of identifying the carotenoid pigments, the chromatographic behaviour, and the UV-visible and mass spectroscopic characteristics were studied for each peak. **Table 1** summarises the identification for each chromatographic peak. **Table 1** includes the quantitative composition of tha sarsaparilla berries, showing a total carotenoid content of about 377 µg/g of fresh weight. Lycopene was the major pigment (242.44 µg/g fw) accounting for up to 64% of the total carotenoid content, and therefore being the main responsible for the red colour of the ripe berries. Othe major carotenoids were β-cryptoxanthin (41.17 µg/g fw; including the free and esterified forms) and β-carotene (65.76 µg/g), followed by lower amounts of lutein, zeaxanthin, rubixanthin and antheraxanthin. **Figure 3** shows the structures of the carotenoids present in sarsaparilla berries. The identification of the native β-cryptoxanthin esters was achieved by using HPLC linked with a photodiode array detector (PDA) and a mass spectrometer detector (Micromass ZMD4000) with an APCI source (Atmospheric Pressure Chemical Ionisation). It is well known that the use of this soft ionization technique allows the simultaneous determination of the fatty acid(s) present in a xanthophyll esters, together with the structural assignment of the carotenoid backbone. In the present work, we identify the entire family of acyl esters of β-cryptoxanthin ranging from capric acid (C10:0) to stearic acid (C18:0). Traces of zeaxanthin monoester were detected but the low concentration level did not allow the complete structural determination. Diesters were not detected for any of the dihydroxylated xanthophylls present in the extracts. It is noteworthy to mention that all the esters contained exclusively saturated fatty acids, whereas in the fatty acid composition of the sarsaparilla berries the major fatty acids were unsaturated ones, myristoleic acid (40.4%) and linoleic acid (23.2%), followed by palmitic acid (18.5%), myristic acid (2.7%) and stearic acid (6.6%). This fact suggests that the enzyme responsible for the formation of xanthophyll esters is highly selective regarding the fatty acid.

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